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(54) Title: IGF-1 ANALOGS

(57) Abstract

Pharmaceutical compositions which comprise short peptides which function as IGF-1 receptor antagonists are provided. The peptides used in the pharmaceutical compositions of the invention consist of less than 25 amino acids, comprise at least a portion of the C or D domain from IGF-1 and inhibit IGF-1 induced autophosphorylation of IGF-1 receptors. Methods of inhibiting cell proliferation and of treating individuals suspected of suffering from or susceptible to diseases associated with undesirable cell proliferation such as cancer, restenosis and asthma are disclosed.

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## IGF-1 ANALOGS

### CROSS REFERENCE TO RELATED PATENT APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial Number 07/881,524 filed May 8, 1993, 5 pending, which is incorporated herein by reference.

### BACKGROUND OF THE INVENTION

Evidence indicates that the interaction of Insulin-like Growth Factor-1 (IGF-1) with its own receptor (IGF-1R or, alternatively referred to as type 1 receptor) plays a major 10 role in normal development and in the control of both normal and abnormal cell growth. In growth hormone disturbances of growth as, for instance, in acromegalics and in patients with growth hormone deficiency, clinical assessments of disease activity correlate far better with blood levels of IGF-1 than 15 they do with growth hormone concentrations (Van Wyk et al., The Biology of Normal Human Growth, pp. 223-239, Raven Press, NY (1981)). Werner et al., *Proc. Nat. Acad. Sci. USA*, 86:7451-5 (1989) have shown that the mRNA levels for the IGF-1R decrease steadily in all tissues during post-natal development, reaching 20 a maximum during the perinatal stages. IGF-1 mRNA, instead, is not so tightly regulated during development as the mRNA for the IGF-1 R, and actually reaches maximum expression in the adult liver, which is the main site of production of IGF-1.

Apart from these general considerations, a number of 25 reports have appeared indicating that the interaction of IGF-1 with its own receptor play a major role in cell growth. For instance, IGF-1Rs are present in phytohemagglutinin activated T lymphocytes, Kozak et al., *Cell Immunol.*, 1009:318-331 (1987)

and in K562 cells that are a human erythroleukemia cell line, Hizuka et al., *Endocrinol. Japon*, 34:81-88 (1987). In fact, K562 cells grow vigorously in serum free media (SFM) containing only IGF-1 or supraphysiological concentrations of insulin. An abundance of IGF-1Rs has also been reported in lymphoblasts of human T cell leukemias, Lee et al., *J. Clin. Endocrinol. & Metabol.*, 62:28-35 (1986), and in HL60 cells, Pepe et al., *J. Cell Physiol.*, 133:219-227 (1987). In our own laboratory, we have been able to show that the mRNA for the IGF-1R is over-expressed in HL60 cells. HL60 cells, as well as other cell lines, grow well in serum-free medium containing only insulin in supraphysiological concentrations. In Burkitt cells, the number of IGF-1Rs increase between G<sub>1</sub> and S-3 phase, Hartman et al., *Leukemia*, 2:241-4 (1988). Stem cells and progenitor cells also seem to require IGF-1 for growth. Goldring and Goldring, *Eucar. Gene Express*, 1:-301-326 (1991), list several references indicating that IGF-1 increases the proliferation of keratinocytes, smooth muscle cells, osteoblasts, chondrocyts and neuronal cells (see their Table 4). The IGF-1R is induced by estrogens in breast cancer cell lines, Stewart et al., *J. Biol. Chem.*, 265:21172-8 (1990), Pekonen et al., *Cancer Res.*, 48:1343-7 (1988), Peyrat et al., *Cancer Res.*, 48:6429-33 (1988), Foekens et al., *Cancer Res.*, 49:5823-8 (1989), and the expression of IGF-1Rs seems to correlate with the growth of breast cancer, at least just as well as the estrogen receptors or the EGF receptor. Other tumors in which an increased expression of IGF-1R or, at least, IGF-1 binding sites, have been reported include small cell lung cancer, Kiefer et al., *Exp. Cell Res.*, 184:396-406 (1989), Minuto et al., *Cancer Res.*, 48:3716-9 (1988), Nakanishi et al., *J. Clin. Invest.*, 82:354-9 (1988), choriocarcinoma cells, Ritvos et al., *Endocrinology*, 122:395-401 (1988), malignant glioma, Gammeltoft et al., *Cancer Res.*, 48:1233-7 (1988), renal carcinoma, Pekonen et al., *Int. J. Cancer*, 43:1029-33 (1989), and neoplastic human endometrium, Talavera et al., *J. Cancer Res.*, 50:3019-24 (1990). A role of the IGF-1R in growth has also been reported in human melanoma

cells, Stracke et al., *J. Biol. Chem.*, 264:21544-9 (1989), and in tumors of neural origins like neuroblastomas or pheochromocytomas, Ota et al., *Molec. Brain Res.*, 6:69-76 (1989) and Ota et al., *Cur. J. Biochem.*, 174:521-30 (1988).

5 However, the best evidence that the IGF-1R plays a major role in the control of cellular proliferation comes from studies with fibroblasts in cell cultures.

When IGF-1 binds to IGF-1R, IGF-1R undergoes autophosphorylation. The autophosphorylation is believed to be  
10 an important event in cell growth and proliferation. Thus, IGF-1 induced autophosphorylation of IGF-1R is believed to be involved in the undesirable cell growth and proliferation involved in the pathogenesis associated with diseases and disorders such as, for example, cancer, restenosis and asthma.

15 There is a need for pharmaceutical compositions which can effectively inhibit the cell proliferation which results from IGF-1R autophosphorylation which normally occurs when IGF-1R bind with IGF-1. There is a need for pharmaceutical compositions which can effectively inhibit the IGF-1 induced  
20 autophosphorylation of IGF-1R which normally occurs when IGF-1R binds with IGF-1. There is a need for a method of inhibiting cell proliferation which results from IGF-1R autophosphorylation which normally occurs when IGF-1R bind with IGF-1. There is a need for a method of inhibiting IGF-1  
25 induced autophosphorylation of IGF-1R which normally occurs when IGF-1R binds with IGF-1. There is a need for a method of treating individuals suspected of suffering from or susceptible to diseases and disorders associated with undesirable cell proliferation.

### 30 SUMMARY OF INVENTION

The present invention relates to pharmaceutical compositions comprising:

- a) a synthetic peptide which comprises
  - i) less than 25 amino acids,
  - ii) a sequence corresponding to at least  
35 a portion of the C or D domain of human insulin-like growth

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factor 1, wherein the peptides inhibit IGF-1 induction of autophosphorylation by IGF-1R; and

b) a pharmaceutically acceptable carrier or diluent.

5           The present invention relates to methods of inhibiting cell proliferation comprising contacting selected cells with a peptide comprising less than 25 amino acids and having a sequence corresponding to at least a portion of the C or D domain of human insulin-like growth factor 1.

10           The present invention relates to methods of treating an individual suspected of suffering from or susceptible to a disease associated with undesirable cell proliferation comprising:

administering an effective amount of a pharmaceutical  
15 composition comprising:

a) a synthetic peptide which comprises  
i) less than 25 amino acids,  
ii) a sequence corresponding to at least  
a portion of the C or D domain of human insulin-like growth  
20 factor 1, wherein the peptides inhibit IGF-1 induction of autophosphorylation by IGF-1R; and  
b) a pharmaceutically acceptable carrier or  
diluent.

#### DESCRIPTION OF THE DRAWINGS

25           Figure 1 shows the peptide sequences and shows the C and D "flaps" on a molecular model of the IGF-1 molecule.

Figure 2 shows the effect of the IGF-1 analog on the growth of p6 cells. p6 cells were cultured in SFM for 48 hours and then treated with different concentrations of JB-IGF-1 only  
30 (bars 6, 7), or IGF-1 at a concentration of 10 ng/ml (bars 8, 11). Cells were counted 48 hours after treatment.

Figure 3 shows the effect of treatment with the peptide JB1. The first band shows unstimulated cells. The second and third bands show the effects of stimulation with  
35 insulin and IGF-1, respectively. The fourth and fifth bands

show the effects of treatment with JB1 at 500 ng/ml and 1000 ng/ml, respectively.

Figure 4 shows the effect of the IGF-1 analog on the growth of p6 cells. p6 cells were treated with JB2 and JB3. 5 The effects on growth of the cells during 48 hours in SFM after stimulation with 20 ng/ml of IGF-1 are shown.

Figure 5 shows the effects of JB4 on growth of p6 cells during 48 hours in SFM. These cells were starved in SFM for 48 hours. The peptide was added directly to conditional 10 medium.

Figure 6 shows the effects of the peptide JB3 on the growth of WI-38 cells in serum-supplemented medium (SSM) during 48 hours. Before treatment with the peptide, the cells were starved in SFM for 48 hours. After treatment, the cells were 15 stimulated with 10% FBS.

Figure 7 shows the effects of treatment with the peptide JB3 on the growth of p6 cells in SSM with JB3 or in SFM with IGF or insulin in the presence of JB3 or JB1 and JB2 during 48 hours

Figure 8 shows the effect of treatment with the peptide JB3 on the growth of DU145 and PC3 cells during 48 hours in culture with serum. Cells were inoculated in SSM and 24 hours later, JB3 was added for 48 hours. 20

#### DETAILED DESCRIPTION OF INVENTION

Human IGF-1 is a 70 amino acid protein that consists of 4 principle domains. The first 29 residues of IGF-1 bear a strong resemblance to the B chain of insulin and, consequently, are known as the B domain. IGF-1 residues 42-62 are homologous to the insulin A chain and hence, known as the A domain. 30 Intervening between the B and A domains (residues 30-41) is the C domain. Finally, the last 7 amino acids (residues 63-70) have been referred to as the D domain. The sequence of IGF-1 is known (SEQ ID NO: 1). Rotwein, P., Pollock, K.M., Didier, D.K., and Krivi, C.C., *J. Biol. Chem.* 261:4828-4832 (1986) 35 (Sequence translated from the DNA sequence); Jansen, M., van Schaik, F.M.A., Ricker, A.T., Bullock, B., Woods, D.E., Gabbay,

K.H., Nussbaum, A.L., Sussenbach, J.S., and Van den Brande, J.L., *Nature* 306:609-611 (1983) (Sequence translated from the mRNA sequence); Met-24 is proposed as a likely initiator. Rinderknecht, E., and Humbel, R.E., *J. Biol. Chem.* 5 253:2769-2776 (1978) (Sequence of residues 49-118).

A detailed solution NMR structure of the core of human IGF-1 was recently reported by Cooke, R.M., Harvey, T.S., Campbell, I.D., *Biochem.*, 30:5484-5491 (1991). The hydrophobic core of IGF-1 is strikingly similar to insulin. In this light, 10 it is interesting to note that, in addition to binding to IGF-1R, IGF-1 also binds the insulin receptor, albeit with lower affinity (Massague, J. and Czech, M.P., *J. Biol. Chem.*, 257:5038-5045 (1982)). The most striking structural differences occur between IGF-1 and an insulin dimer because of 15 the inclusion of the C and D domains in the IGF-1 structure. Both the C and D domains were poorly resolved in the structures due to their intrinsic mobility.

IGF-1 binding to IGF-1R induces autophosphorylation of IGF-1R which activates IGF-1R. Activated IGF-1R is 20 associated with cellular growth and proliferation. In diseases and disorders characterized by undesirable cell growth and proliferation such as, for example, cancer, restenosis and asthma, inhibition of IGF-1R autophosphorylation is desirable as a means of preventing IGF-1R activation. Thus, 25 pharmaceutical compositions useful to treat diseases characterized by cell growth and proliferation are desirable and may include compounds which inhibit the activation of IGF-1R that occurs when the receptor binds with IGF-1. Such compounds may prevent binding or allow binding but prevent 30 induction of autophosphorylation of IGF-1R by IGF-1.

A molecular model of the human IGF-1 (for general details regarding the building of this molecular model (see, Jameson, B.A., *Nature*, 341:465-466 (1989) which is incorporated herein by reference) that is consistent with the NMR data 35 obtained by Cooke et al. (1991) (*supra*) has been developed. In this model, the C and D domains appear as "flaps" which flank the insulin-conserved receptor binding cleft (residues 21-24,



Cascieri, M.A., Chicchi, G.G., Applebaum, J., Hayes, N. Green, B.C., Bayne, M.L., *Biochem.*, 27:3229-3233 (1988); Bayne, M.L., Applebaum, J., Underwood, D., Chicchi, G.G., Green, B.C., Hayes, N., Cascieri, M.A., *J. Biol. Chem.*, 264:11004-11008  
5 (1989). Evidence indicates that these flaps are directly involved in the specific binding to IGF-1R. It has been observed that deletion of the D domain of IGF-1 increased the affinity of the mutant IGF-1 for binding to the insulin receptor, while decreasing its affinity for the IGF-1R  
10 (Cascieri et al., 1988) (*supra*). Furthermore, some or all of the residues within the C domain, which flank the conserved binding cleft in IGF-1, but not in insulin, appear to be required for distinguishing between the IGF-1R and insulin receptors (Bayne et al., (1989) (*supra*); Cascieri, M.A. and  
15 Bayne, M.L., Molecular and Cellular Biology of IGFs and Their Receptors, LeRoth, D. and Raizada, M.K., Eds., Plenum Press (London 1990).

Evidence is provided herein showing that the C and D "flaps" of IGF-1 are involved with the highly specific binding  
20 of this protein to IGF-1R. Peptides which comprise at least a portion of either flap may be used to inhibit IGF-1 induction of IGF-1R autophosphorylation. Targeting the C and D domains of IGF-1 for synthetic analog design has yielded highly specific competitive inhibitors of IGF-1R binding. In  
25 particular, the C domain (residues 30-41) and D domain (residues 63-70) and fragments thereof, alone or linked to non-IGF-1 sequences have been selected for peptide mimicry. Further, analogs which do not completely inhibit IGF-1/IGF-1R binding may inhibit autophosphorylation of IGF-1R nonetheless.

30 The methods of the present invention include methods of treating individuals suffering from or susceptible to diseases and disorders characterized by cell proliferation comprising the steps of administering to an individual an effective amount of a peptide which inhibits IGF-1 induced  
35 autophosphorylation of IGF-1R. These diseases and disorders include, but are not limited to, restenosis of the coronary arteries after angioplasty, human neoplasia such as cancer of

the prostate, tumors in pleural and peritoneal cavities and brain metastases, smooth muscle cell hyperplasia in asthma, burns and wounds, and bone marrow containing highly proliferating cells. Diagnosis of diseases and disorders involving undesirable proliferation of cells such as those diseases and disorders outlined above may be made those having ordinary skill in the art. Likewise, identification of those individuals susceptible to such diseases and disorders is also within the routine ability of those having ordinary skill in the art. For example, methods of diagnosing cancer and asthma are well known. In the case of a method of preventing restenosis, an individual to whom angioplasty is to be performed may be treated. Pharmaceutical compositions useful in the methods of the present invention are defined below.

15           Peptides of less than 25 amino acids are provided comprising an amino acid sequence corresponding to at least a portion of the C or D domain of the human insulin-like growth factor 1. These peptides have a restricted conformation and the ability to inhibit the induction of IGF-1R  
20 autophosphorylation by natural IGF-1, thereby inhibiting the action of the IGF-1R. The inhibition of autophosphorylation of IGF-1R can be used as the mechanism to prevent or treat diseases and disorders associated with undesirable and/or abnormal growth and proliferation of cells.

25           The present invention provides pharmaceutical compositions that comprise synthetic peptides of less than 25 amino acids which comprise at least a portion of the IGF-1 C or D domain of IGF-1 and which are capable of inhibiting the induction of IGF-1R autophosphorylation by natural IGF-1 and  
30 thereby cell proliferation. These synthetic peptides are also interchangeable referred to herein as IGF-1 analogs, synthetic analogs or analogs.

          Using the amino acid sequence of IGF-1, and the molecular modelling described above, we have synthesized short  
35 peptides which function as analogs of IGF-1. The effects of treatment with these peptides are shown in Figures 2-8. A peptide that was synthesized to comprise the last 12 amino

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acids of the IGF-1 sequence, when used at nanogram concentrations, completely abolishes the proliferation of a variety of cells. The peptide is non-toxic; i.e., the cells exposed to it remain viable for long periods of time. The inhibitor effect is also reversible, i.e., when the peptide is removed and growth factors are added again, the cells resume proliferation. Inhibition is close to 100%, and it should apply to all cells that require the IGF-1/IGF-1 receptor interaction for growth. These cells include the following: fibroblasts, smooth muscle cells, chondrocytes and osteoblasts, hemopoietic cells of various lineages and keratinocytes. Several of these cell types have been actually tested, and the inhibition by the IGF-1 analog is efficient (almost 100%), and reproducible. For instance, when using the IGF-1 analog, we have inhibited the growth of fibroblasts, and fibroblast-like cells, of T-lymphocytes and of epithelial cells derived from carcinoma of the prostate.

Initial synthetic analogs were designed to incorporate at least a portion of the amino acid sequence of the C and D domains of IGF-1. An attempt was made to maintain the distance geometries and torsional properties of the initial dihedral angles of the domains as they "bud-off" of the hydrophobic protein core. Experimental evidence as well as theoretical calculations indicate strong conformational flexibility of these domains. In order to maximize the overlap between the conformational repertoire of the native protein with that of the synthetic analogs, the analogs have been circulized via an artificially introduced disulfide bridge. With these restraints, the rest of the amino acid sequence of the domain should adopt a folding pattern similar to that imparted by the native structure.

The present invention provides pharmaceutical compositions that comprise peptides which are less than 25 amino acids. It is preferred that peptides are as small as possible. In some embodiments, the peptides are about 4-20 amino acids. In some embodiments, the peptides are about 4-12 amino acids. Amino acid sequences of peptides of the present

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invention comprise a portion of the C or D domain of IGF-1 that is at least 2-7 amino acids of the C domain or at least 2-12 amino acids of the D domain.

The present invention provides pharmaceutical compositions that comprise peptides which contain at least a portion of the IGF-1 C or D domain of IGF-1. The portion of the C or D domain of IGF-1 may be from 2 amino acids to the complete domain (a complete C is 12 amino acids; a complete D domain is 7 amino acids). Non-IGF-1 amino acid sequences are provided in some embodiments. In other embodiments, the peptide contains only IGF-1 amino acid sequences. At least 50% of the amino acid sequence of the peptides of the present invention are preferably derived from the portion of the C or D domain of IGF-1 in some embodiments of the invention. In some embodiments which comprise more than two IGF-1 derived amino acid residue sequences, it is preferred that greater than about 20-25% of the amino acid sequence of the peptides of the present invention are preferably derived from the portion of the C or D domain of IGF-1, more preferably 30-40% and more preferably greater than 50%. In some embodiments, the percentage of amino acid sequence of the peptides of the present invention derived from the portion of the C or D domain of IGF-1 approaches about 60% or about 75% or more.

The present invention provides pharmaceutical compositions that comprise peptides which are capable of inhibiting the induction of IGF-1R autophosphorylation by natural IGF-1 and thereby cell proliferation. Example 1 contains a cell proliferation assay which can be used by one having ordinary skill in the art to test whether a peptide has cell proliferation inhibitory activity. Induction of IGF-1R autophosphorylation and inhibition of induction may be carried out essentially by the method of Lammers et al. (1989) *EMBO J.* 8:1369-1375, which is incorporated herein by reference, using the monoclonal antibody to IGF-1R (Oncogene Sciences, Uniondale, NY), an anti-phosphotyrosine antibody (UBI, Saranac Lake, NY) and the advanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

The peptides of the present invention may be prepared by any of the following known techniques. Conveniently, the peptides may be prepared using the solid-phase synthetic technique initially described by Merrifield, in *J. Am. Chem. Soc.*, 15:2149-2154 (1963). Other peptide synthesis techniques may be found, for example, in M. Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed. (1976); Kent and Clark-Lewis in Synthetic Peptides in Biology and Medicine, p. 295-358, eds. Alitalo, K., Partanen, P. and Vakeri, A., Elsevier Science Publishers, (Amsterdam, 1985); as well as other reference works known to those skilled in the art. A summary of peptide synthesis techniques may be found in J. Stuart and J.D. Young, Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford, IL (1984). The synthesis of peptides by solution methods may also be used, as described in The Proteins, Vol. II, 3d Ed., p. 105-237, Neurath, H. et al., Eds., Academic Press, New York, NY (1976). Appropriate protective groups for use in such syntheses will be found in the above texts, as well as in J.F.W. McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, NY (1973).

In general, these synthetic methods involve the sequential addition of one or more amino acid residues or suitable protected amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively-removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group, such as lysine.

Using a solid phase synthesis as an example, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added

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amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to provide the final peptide. The peptide of the invention are preferably devoid of benzylated or methylbenzylated amino acids. Such protecting group moieties may be used in the course of synthesis, but they are removed before the peptides are used. Additional reactions may be necessary, as described elsewhere, to form intramolecular linkages to restrain conformation.

The present peptides may also be prepared by recombinant DNA techniques, although such methods are not preferred because of the need for purification and subsequent chemical modifications to conformationally restrain the peptides.

In addition to peptides which comprise L amino acids, pharmaceutical compositions according to the present invention may comprise peptides made up of D amino acids. Because most enzymes involved in degradation recognize a tetrahedral alpha-carbon, the D-amino acids were utilized in order to avoid enzyme recognition and subsequent cleavage. Our computer studies indicate that the same folded presentation of the peptide is accomplished by reversing the amino acid sequence, employing D-amino acids. Thus, peptides comprised of D amino acids are less susceptible to degradation.

Conservative substitutions in the amino acid sequence may be made. Those having ordinary skill in the art can readily design IGF-1 analogs with conservative substitutions for amino acids. For example, following what are referred to as Dayhof's rules for amino acid substitution (Dayhof, M.D. (1978) *Nat. Biomed. Res. Found.*, Washington, D.C. Vol. 5, supp. 3), amino acid residues in a peptide sequence may be substituted with comparable amino acid residues. Such substitutions are well known and are based the upon charge and structural characteristics of each amino acid.

Peptides useful in the pharmaceutical compositions of the present invention are designed using IGF-1 sequence (SEQ ID NO:1) information, particularly sequences which make up the C (30-41) and D (63-70) domains. Peptides of less than 25 amino acids total which comprise at least 2 amino acid residues derived from the C or D domain are synthesized. In peptides having only 2-3 amino acid residue sequences that are portions of the C or D domain, the peptide is preferably 4-8 amino acids total. In peptides having 4 or more amino acid residue sequences that are portions of the C or D domain, the peptide is preferably 4-15, more preferably 4-12 amino acids total. L or D amino acids may be used in the synthesis. Peptides may be synthesized with amino acid sequences in the order they occur in IGF-1 or in the reverse order. In peptides comprising all L amino acids, it is preferred that they are synthesized such that the amino acid sequences are assembled in the order that they occur in IGF-1. In peptides comprising all D amino acids, it is preferred that they are synthesized such that the amino acid sequences are assembled in the reverse order that they occur in IGF-1.

Synthesized peptides which contain portions of the C or D domain may be circularized in order to mimic the geometry of those portions as they occur in IGF-1. Circularization may be facilitated by disulfide bridges between cysteine residues. Cysteine residues may be included in positions on the peptide which flank the portions of the peptide which are derived from IGF-1 C or D domain. Cysteine residues within the portion of the peptide derived from IGF-1 C or D domain may be deleted and/or conservatively substituted to eliminate the formation of disulfide bridges involving such residues. Alternatively, the peptides may be circularized by means of covalent bonds, such as amide bonds, between amino acid residues of the peptide such as those at or near the amino and carboxy termini.

Peptides in some embodiments consists of SEQ ID NO: 3. Peptides in some embodiments comprise SEQ ID NO: 3. Peptides in some embodiments consists of fragments of SEQ ID

NO: 3. Peptides in some embodiments comprise fragments of SEQ ID NO: 3.

Peptides in some embodiments consists of D amino acid peptide JB3, described below. Peptides in some embodiments  
5 comprise JB3. Peptides in some embodiments consists of fragments of JB3. Peptides in some embodiments comprise fragments of JB3.

Peptides in some embodiments consists of SEQ ID NO:  
4. Peptides in some embodiments comprise SEQ ID NO: 4.  
10 Peptides in some embodiments consists of fragments of SEQ ID NO: 4. Peptides in some embodiments comprise fragments of SEQ ID NO: 4.

Peptides in some embodiments consists of D amino acid-containing peptide JB2, described below. Peptides in some  
15 embodiments comprise JB2. Peptides in some embodiments consists of fragments of JB2. Peptides in some embodiments comprise fragments of JB2.

Peptides in some embodiments consists of SEQ ID NO:  
6. Peptides in some embodiments comprise SEQ ID NO: 6.  
20 Peptides in some embodiments consists of fragments of SEQ ID NO: 6. Peptides in some embodiments comprise fragments of SEQ ID NO: 6.

Peptides in some embodiments consists of D amino acid peptide Cys Lys Ser Cys. Peptides in some embodiments comprise  
25 D amino acid peptide Cys Lys Ser Cys. Peptides in some embodiments consists of fragments of Cys Lys Ser Cys. Peptides in some embodiments comprise fragments of Cys Lys Ser Cys.

In particular, peptides in some embodiments comprise D amino peptide Lys Ser, preferably with additional components  
30 to provide the correct spatial geometry.

Peptides for use in pharmaceutical compositions of the present invention may be designed following the guidelines set out herein and using well known processes. Methods of synthesizing peptides and circularizing them may be performed  
35 routinely using standard techniques and readily available starting materials.



To determine whether a peptide having the structural properties defined herein is useful in the pharmaceutical compositions and methods of the present invention, routine assays may be performed using such peptides to determine  
5 whether the peptides possess the requisite activity; i.e. whether the peptide can inhibit IGF-1 induced autophosphorylation of IGF-1R. The peptides ability to inhibit cell proliferation may be determined by observing its activity in a cell proliferation assay. As noted above, induction of  
10 IGF-1R autophosphorylation and inhibition of induction may be carried out essentially by the method of Lammers et al. (1989) *EMBO J.* 8:1369-1375, which is incorporated herein by reference, using the monoclonal antibody to IGF-1R (Oncogene Sciences, Uniondale, NY), an anti-phosphotyrosine antibody (UBI, Saranac  
15 Lake, NY) and the advanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Example 1 contains a cell proliferation assay which can be used by one having ordinary skill in the art to test whether a peptide has cell proliferation inhibitory activity.

20 Accordingly, peptides having the structural characteristics described above may be synthesized routinely. Such peptides may be tested using standard assays to determine if they can be used in pharmaceutical compositions and methods according to the present invention.

25 The pharmaceutical composition of the present invention may be formulated by one having ordinary skill in the art with compositions selected depending upon the chosen mode of administration. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, A. Osol, a  
30 standard reference text in this field.

For parenteral administration, the IGF-1 analog can be, for example, formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are  
35 water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The vehicle or lyophilized powder

may contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques. For example, a parenteral composition suitable for 5 administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

The pharmaceutical compositions according to the present invention may be administered as a single dose or in 10 multiple doses. The pharmaceutical compositions of the present invention may be administered either as individual therapeutic agents or in combination with other therapeutic agents. The treatments of the present invention may be combined with conventional therapies, which may be administered sequentially 15 or simultaneously.

The pharmaceutical compositions of the present invention may be administered by any means that enables the active agent to reach the targeted cells. Because peptides are subject to being digested when administered orally, parenteral 20 administration, i.e., intravenous, subcutaneous, intramuscular, would ordinarily be used to optimize absorption. Intravenous administration may be accomplished with the aid of an infusion pump. The pharmaceutical compositions of the present invention may be formulated as an emulsion. Alternatively, they may be 25 formulated as aerosol medicaments for intranasal or inhalation administration. In some cases, topical administration may be desirable.

The dosage administered varies depending upon factors such as: pharmacodynamic characteristics; its mode and route of 30 administration; age, health, and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment; and frequency of treatment. Usually, the dosage of peptide can be about 1 to 3000 milligrams per 50 kilograms of body weight; preferably 10 to 1000 milligrams per 50 kilograms of body 35 weight; more preferably 25 to 800 milligrams per 50 kilograms of body weight. Ordinarily 8 to 800 milligrams are administered to an individual per day in divided doses 1 to 6

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times a day or in sustained release form is effective to obtain desired results.

Depending upon the disease or disorder to be treated, the pharmaceutical compositions of the present invention may be formulated and administered to most effectively inhibit undesirable cell proliferation.

Restenosis is a side effect often occurring when balloon angioplasty is performed. The disruption of the endothelial lining of the blood vessel being cleared by angioplasty exposes the underlying smooth muscle cells. Undesirable smooth muscle cell proliferation occurs when the exposed smooth muscle cells proliferate absent the contact inhibition that occurs when the endothelial lining is present. The proliferating cells congest the blood vessel resulting in restenosis. Much of the undesirable proliferation occurs within the first 24 hours after angioplasty. Thus, when used in conjunction with angioplasty, the pharmaceutical composition is administered one or more times within the first 24 hours post angioplasty. The pharmaceutical compositions of the present invention may be formulated as an emulsion which can be delivered with the balloon catheter to the area where angioplasty is to take place.

In individuals suffering from asthma, cells of the lungs chronically proliferate and congest the lung. The pharmaceutical compositions of the present invention may be formulated as an aerosol. Such an aerosol medicament may be administered periodically. In individuals suffering from cancer, cells such as tumor cells proliferate chronically. The pharmaceutical compositions of the present invention may be administered periodically. In addition, the pharmaceutical compositions of the present invention may be injected at a site at or near hyperproliferative growth. For example, administration may be by direct injection into a solid tumor mass or in the tissue directly adjacent thereto.

The invention is further illustrated by means of the following, non-limiting examples.

**EXAMPLES****Example 1**

The first peptide synthesized represents the looped-out region of the D domain, residues 61-69. The native  
5 sequence from 61-70 is represented as SEQ ID NO: 2.

SEQ ID NO: 2: Met Ala Pro Leu Lys Pro Ala Lys Ser Ala

The synthesized peptide, SEQ ID NO: 3, also designated JB1, includes residues 61-69 plus additional non-IGF-1 residues including Cysteines used to form a disulfide bridge to maintain  
10 the geometry of the peptide.

SEQ ID NO: 3 (JB1): Cys Tyr Ala Ala Pro Leu Lys Pro Ala  
Lys Ser Cys

The distance from Met-60 to Ala-70 spans ~ 6.0 Å, as measured in our molecular model. This distance and geometry  
15 can be maintained by the use of a disulfide bridge. The Cysteine at position 62 was replaced with an alanine in order to avoid an inappropriate disulfide linkage. The peptide was synthesized using L-amino acids and standard solid phase peptide synthesis. It should be noted that the analog JB1,  
20 although a potent antagonist of the IGF-1 receptor activity, suffers from rapid degradation in sera.

**Example 2**

In another embodiment, the L-amino acids of JB1 (SEQ ID NO:3) were substituted with D-amino acids. Because most  
25 enzymes involved in degradation recognize a tetrahedral alpha-carbon, the D-amino acids were utilized in order to avoid enzyme recognition and subsequent cleavage. Our computer studies indicate that the same folded presentation of the peptide is accomplished by reversing the amino acid sequence, employing D-amino acids. The following D-amino acid peptide,  
30 designated JB3 was synthesized using standard solid phase techniques and D amino acid residues as starting materials.

D amino acid peptide JB3:

Cys Ser Lys Ala Pro Lys Leu Pro Ala Ala Tyr Cys

**Example 3**

In yet another embodiment, the distance from Ala-62 to Ala-70 spans ~ 7.5 Å. This distance and geometry can be maintained by the use of a tBoc/fMoc protection strategy to mediate the formation of an amide bond between the amino and carbon termini of the peptide. The peptide will be internally cross-linked via the side chains of a lysine (epsilon amino group) and the carboxylic acid function of an aspartic acid side chain, thus creating an amide bond. Consequently, Ala-62 and Ala-70 will be replaced with Asp and Lys, respectively. The peptide is synthesized according to standard procedures on a low substitution (0.2 mM/gm or less) paramethylbenzhydrylamine resin. The first residue added to the resin is an N-alpha-tBOC, epsilon-fMOC lysine. The rest of the peptide is continued normally using tBOC chemistry until the final residue is added. The last residue to be added is a Z-protected glutamic acid, in which the carboxylic acid moiety is protected with a tert-butyl group. Treatment of the peptide-resin with piperidine/DMF removes the fMOC group from the epsilon amino group of the initial lysine without affecting any other protection groups, and subsequent treatment with TFA removes the protection of the carboxylic acid group of the aspartic acid. Following neutralization, the peptide is covalently closed using a standard diimide-mediated coupling reaction. It should be emphasized that this is only one of the ways in which the synthetic peptide can be covalently closed, however, this method will give a flexible connection at the ends of the peptide that spans a distance of ~ 7.5 Å from the alpha carbon of the Asp to the alpha carbon of the Lys.

The amino acid sequence of this derivative peptide, which is covalently linked by an amide bond between Asp and Lys is designated SEQ ID NO:4.

Asp Pro Leu Lys Pro Ala Lys Ser Lys

**Example 4**

Another peptide represents the looped-out region of the C domain, residues 29-38:

Thr Gly Tyr Gly Ser Ser Ser Arg Arg (SEQ ID NO: 5).

- 5 In order to maintain flexibility, torsional properties and distance, a Cys-Gly was placed on the amino terminus of the peptide and a Cys(D) was placed on the carboxy terminus. Thus, the D amino acid-containing peptide derivative of SEQ ID NO:5 is JB2 Cys Gly Thr Gly Tyr Gly Ser Ser Ser Arg Arg Cys(D)

**10 Example 5**

- In yet another embodiment, a small piece of the insulin-like core was included in this sequence in order to maintain the correct geometry. The distance spanned here represents ~ 10 Å. Because this is a fixed planar geometry, a  
15 toluene-based derivative will be employed to mimic the spatial properties of the native protein. An fMoc/tBoc strategy will be used to create a planar, covalent closure of the peptide between free amino groups at the ends of the peptide utilizing  
20 cross-linker. The methyl group of the aromatic ring of TDI prevents the isocyanate group in the 2 position from reacting at a pH 7.5 or below, whereas the isocyanate group in the para position is highly reactive. A shift in pH to greater than 9.0  
25 will initiate a reaction with the isocyanate group in the 2 position, thus enabling highly specific and controlled conditions for a 10 Å covalent closure of the peptide.

**Example 6**

- In another embodiment, SEQ ID NO: 6 was constructed which contains Cys Lys Ser Cys. This four residue peptide was  
30 circularized.

A D amino acid circular peptide was constructed using this sequence.

D amino acid peptide Cys Lys Ser Cys

**Example 7**

In order to determine whether the sequence order is essential for peptide activity, an L amino acid sequence, designated JB4, was constructed which is the reverse sequence of JB1. Thus, JB4 is SEQ ID NO: 7.

SEQ ID NO:7 (JB4) Cys Ser Lys Ala Pro Lys Leu Pro Ala Ala Tyr  
Cys

**Example 8 Effect of IGF-1 Analog on the Growth of p6 Cells**

p6 cells are derived from BALB/c3T3 and have a constitutively over-expressed IGF-1 receptor. Because of the constitutive over-expression of the IGF-1 receptor these cells, although incapable of growing in serum-free medium, can grow very happily when IGF-1 is added to the medium. In Figure 2 we show the effect that the peptide had on the growth of p6 cells stimulated by IGF-1. The first bar is the number of 3T3 cells that were replated. The second bar, actually No. 3, is the number of 3T3 cells 48 hours after plating. 4, 5 and 6 represent the number of 3T3 cells 48 hours later, after stimulation with IGF-1, respectively 5 ng/ml, 10 ng/ml, 20 ng/ml. Bars 8 and 9 show the number of 3T3 cells 48 hours after exposure to the peptide analog at a concentration of 1000 ng/ml, or 100 ng/ml respectively. Note the lack of toxicity. Bars 11, 12, 13 and 14 refer to the number of 3T3 cells stimulated with 10 ng/ml of IGF-1 in the presence of the IGF-1 analog at concentrations of, respectively, 50, 100, 500 and 1000 ng/ml.

Figure 3 shows that the analog completely inhibits the autophosphorylation of the IGF-1 receptor.

**Example 9**

The experiments in Example 8 used as competitive analog the D domain peptide. Figure 4 shows another experiment in which IGF-1-stimulated p6 cells were exposed to JB2 (C domain peptide) and JB3 (D domain peptide, but with D-amino acids). JB2 inhibits approximately 80% at 1  $\mu$ g/ml, whereas JB3 completely inhibits at 500 ng/ml. The effect of a scrambled

peptide (JB4, same amino acid composition as JB1 but in reverse order) is shown in Figure 5. JB4 has no effect on the growth of IGF-1-stimulated p6 cells, indicating that the effect of JB1 is sequence specific.

#### 5 Example 10

In these experiments, the inhibitory effect of JB3 (D-amino acids) was tested in serum-supplemented medium. Figure 6 shows its effect on WI-38 human diploid fibroblasts: JB3 completely suppresses the growth of these cells at 500 ng/ml. 10. The same results were obtained with p6 cells (Figure 7) and with two lines of prostatic carcinoma cells, DU48 and PC3 (Figure 8).

In summary, the following peptides have been found to exert a profound inhibitory effect on the growth of cells in 15 culture: JB1 (D-domain peptide), JB2 (C-domain peptide) and JB3 (D-domain with D-amino acids). These peptides are non-toxic at the concentrations used (up to 5  $\mu$ g/ml) and their effect is reversible. They inhibit a variety of cell types, as predicted from the finding that the activation of the IGF-1 20 receptor is required for the growth of many normal and transformed cells.

The inhibitory effect is sequence specific and JB3 is also active in the presence of serum.

#### Example 11

25 A pharmaceutical composition is formulated which includes a peptide according to SEQ ID NO:3 in phosphate buffered saline. An 800 milligram dose of peptide in this composition is delivered with an infusion pump to an individual within 24 hours after balloon angioplasty is performed.



**Example 12**

A pharmaceutical composition is formulated which includes the D amino acid peptide designated JB3 in phosphate buffered saline. An 800 milligram dose of peptide in this  
5 composition is delivered with an infusion pump to an individual within 24 hours after balloon angioplasty is performed.

**Example 13**

A pharmaceutical composition is formulated which includes a peptide according to SEQ ID NO:4 in phosphate  
10 buffered saline. An 800 milligram dose of peptide in this composition is delivered with an infusion pump to an individual within 24 hours after balloon angioplasty is performed.

**Example 14**

A pharmaceutical composition is formulated which  
15 includes the D amino acid-containing peptide designated JB2 in phosphate buffered saline. An 800 milligram dose of peptide in this composition is delivered with an infusion pump to an individual within 24 hours after balloon angioplasty is performed.

**20 Example 15**

A pharmaceutical composition is formulated which includes a peptide according to SEQ ID NO:6 in phosphate buffered saline. An 800 milligram dose of peptide in this  
25 composition is delivered with an infusion pump to an individual within 24 hours after balloon angioplasty is performed.

**Example 16**

A pharmaceutical composition is formulated which includes the D amino acid peptide Cys Lys Ser Cys in phosphate buffered saline. An 800 milligram dose of peptide in this  
30 composition is delivered with an infusion pump to an individual within 24 hours after balloon angioplasty is performed.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Bradford A. Jameson and Renato Baserga

(ii) TITLE OF INVENTION: IGF-1 Analogs

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Woodcock Washburn

Kurtz Mackiewicz & Norris

(B) STREET: One Liberty Place - 46th Floor

(C) CITY: Philadelphia

(D) STATE: PA

(E) COUNTRY: USA

(F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT 5.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/881,524

(B) FILING DATE: 08-MAY-92,

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Mark DeLuca

(B) REGISTRATION NUMBER: 33,229



- 26 -

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Pro Leu Lys Pro Ala Lys Ser Ala  
1                   5                   10

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Cys Tyr Ala Ala Pro Leu Lys Pro Ala Lys Ser Cys  
1                   5                   10

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Asp Pro Leu Lys Pro Ala Lys Ser Lys  
1                   5

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

- 27 -

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Thr Gly Tyr Gly Ser Ser Ser Arg Arg  
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Cys Lys Ser Cys  
1

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Cys Ser Lys Ala Pro Lys Leu Pro Ala Ala Tyr Cys  
1 5 10

## CLAIMS

What is claimed is:

1. A pharmaceutical composition comprising:
  - a) a synthetic peptide which comprises
    - 5 i) less than 25 amino acids, and
    - ii) a sequence corresponding to at least a portion of the C or D domain of human insulin-like growth factor 1, wherein said peptide inhibits IGF-1 induction of autophosphorylation by IGF-1R; and
  - 10 b) a pharmaceutically acceptable carrier or diluent.
2. The pharmaceutical composition of claim 1 wherein said peptide comprises a sequence of at least 4 amino acid residues corresponding to at least a portion of the C or  
15 D domain of human insulin-like growth factor 1.
3. The pharmaceutical composition of claim 1 wherein said peptide comprises a sequence of at least 7 amino acid residues corresponding to at least a portion of the C or D domain of human insulin-like growth factor 1.
- 20 4. The pharmaceutical composition of claim 1 wherein said peptide comprises a sequence corresponding to at least a portion of the C domain of human insulin-like growth factor 1.
5. The pharmaceutical composition of claim 1  
25 wherein said peptide comprises a sequence corresponding to at least a portion of the D domain of human insulin-like growth factor 1.
6. The pharmaceutical composition of claim 1 wherein at least 50% of amino acid residues of said peptide  
30 consists of a sequence corresponding to at least a portion of the C or D domain of human insulin-like growth factor 1.

7. The pharmaceutical composition of claim 1 wherein said peptide comprises at least one D amino acid residue.

35 8. The pharmaceutical composition of claim 1 wherein said peptide consists of SEQ ID NO: 3.

9. The pharmaceutical composition of claim 1 wherein said peptide consists of D amino acid peptide JB3: Cys Ser Lys Ala Pro Lys Leu Pro Ala Ala Tyr Cys.

40 10. The pharmaceutical composition of claim 1 wherein said peptide consists of SEQ ID NO: 4.

11. The pharmaceutical composition of claim 1 wherein said peptide consists of D amino acid-containing peptide JB2: Cys Gly Thr Gly Tyr Gly Ser Ser Ser Arg Arg  
45 Cys(D).

12. The pharmaceutical composition of claim 1 wherein said peptide consists of SEQ ID NO: 6.

13. The pharmaceutical composition of claim 1 wherein said peptide consists of D amino acid peptide: Cys Lys  
50 Ser Cys.

14. A method of inhibiting cell proliferation comprising contacting selected cells with a peptide comprising less than 25 amino acids and having a sequence corresponding to at least a portion of the C or D domain of human insulin-  
55 like growth factor 1.

15. A method of treating an individual suspected of suffering from or susceptible to a disease associated with undesirable cell proliferation comprising:  
administering an effective amount of a  
60 pharmaceutical composition comprising:

- a) a synthetic peptide which comprises
- i) less than 25 amino acids,
  - ii) a sequence corresponding to at least a portion of the C or D domain of human insulin-like growth factor 1, wherein said peptide inhibits IGF-1 induction of autophosphorylation by IGF-1R; and
- b) a pharmaceutically acceptable carrier or diluent.

16. The method of claim 15 wherein said disease is cancer, restenosis or asthma.

17. The method of claim 15 wherein said peptide consists of SEQ ID NO: 3.

18. The method of claim 15 wherein said peptide consists of D amino acid peptide JB3: Cys Ser Lys Ala Pro Lys Leu Pro Ala Ala Tyr Cys.

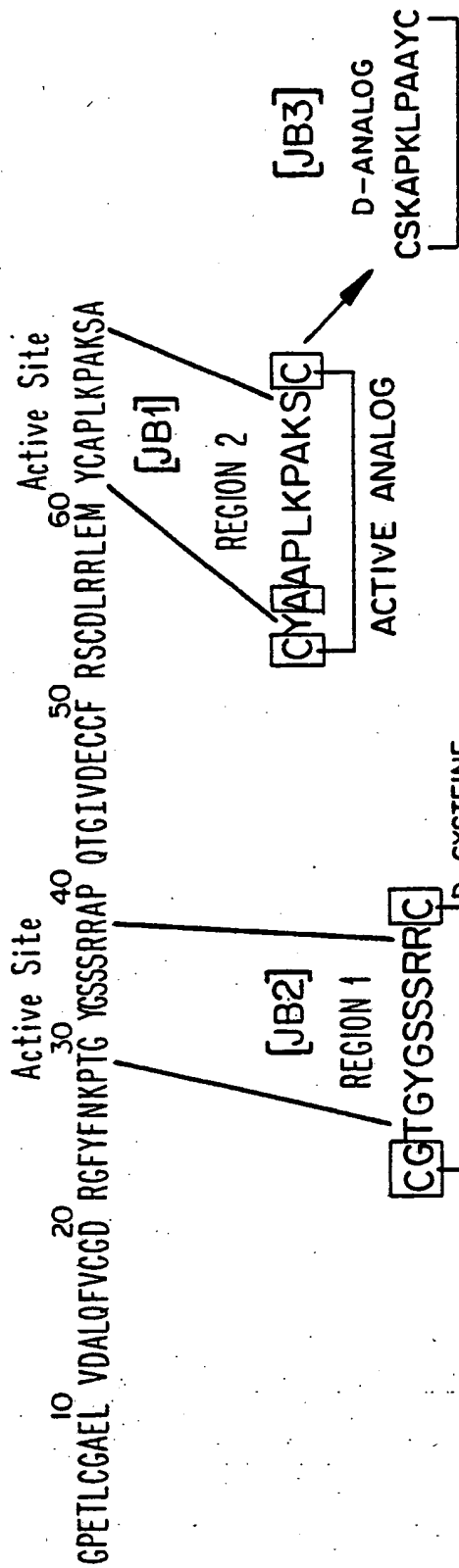
19. The method of claim 15 wherein said peptide consists of SEQ ID NO: 4.

20. The method of claim 15 wherein said peptide consists of D amino acid-containing peptide JB2: Cys Gly Thr Gly Tyr Gly Ser Ser Ser Arg Arg Cys(D).

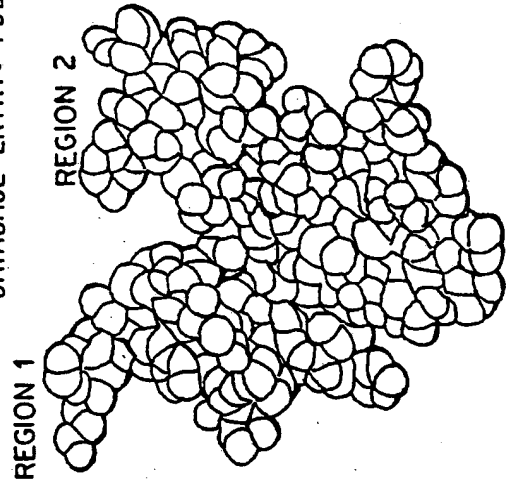
21. The method of claim 15 wherein said peptide consists of SEQ ID NO: 6.

22. The method of claim 15 wherein said peptide consists of D amino acid peptide: Cys Lys Ser Cys.

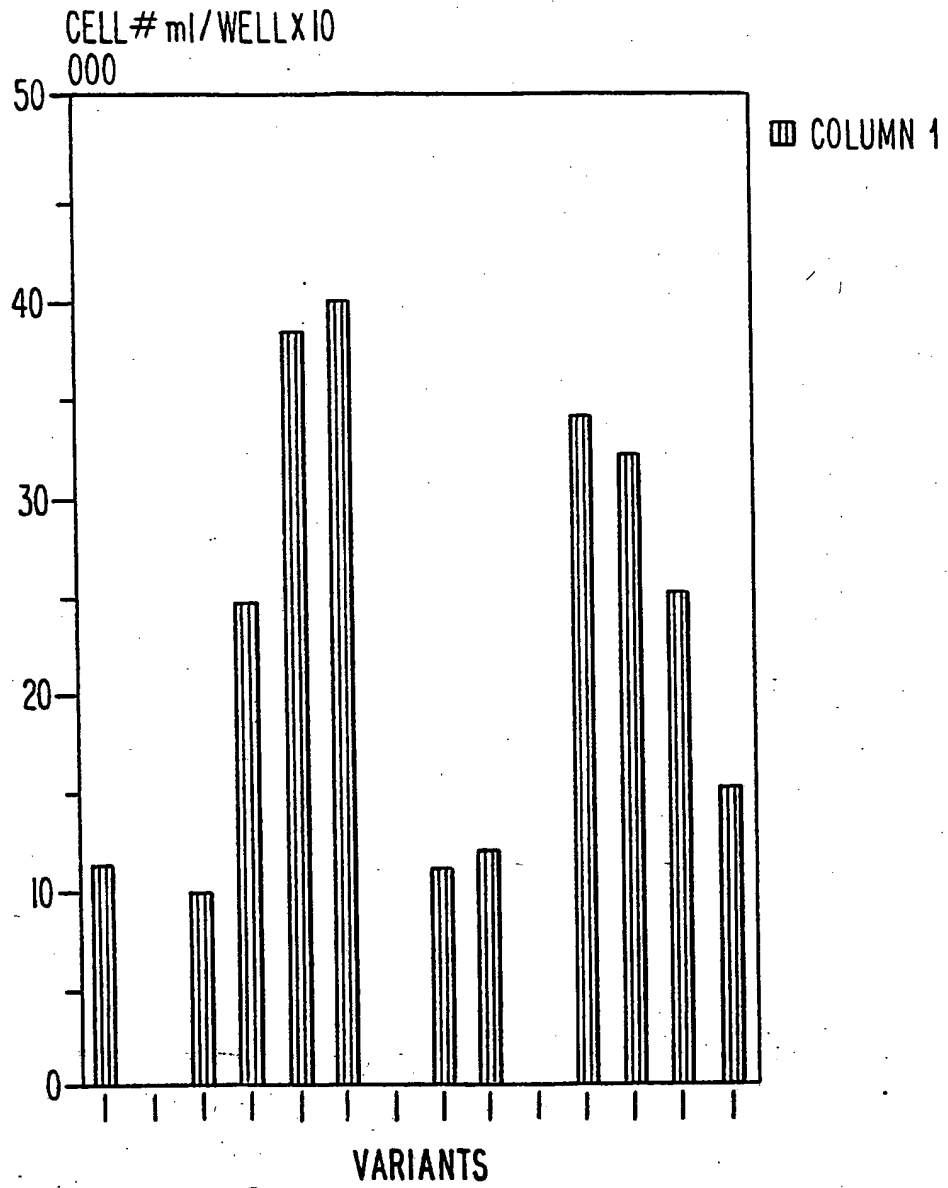




HUMAN IGF 1  
 TAKEN FROM THE BROOKHAVEN  
 DATABASE ENTRY: PDB2GF1.ENT



***Fig. 1***



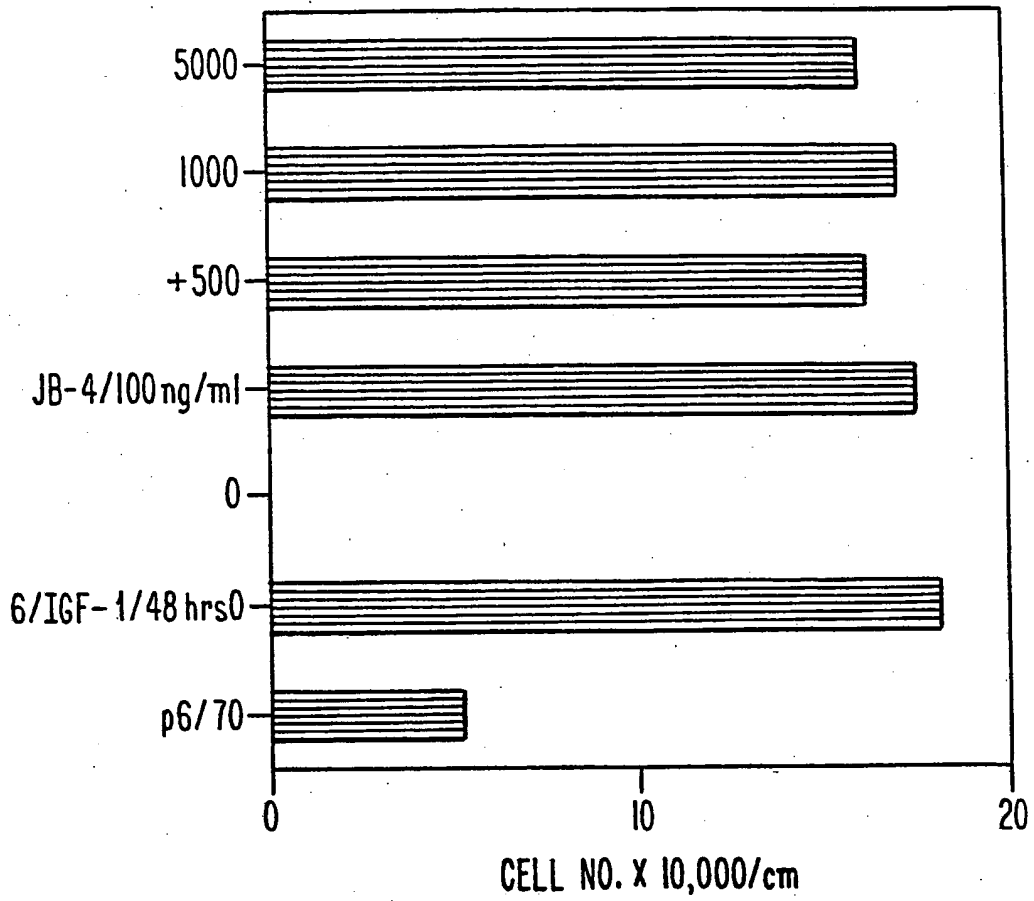
***Fig. 2***



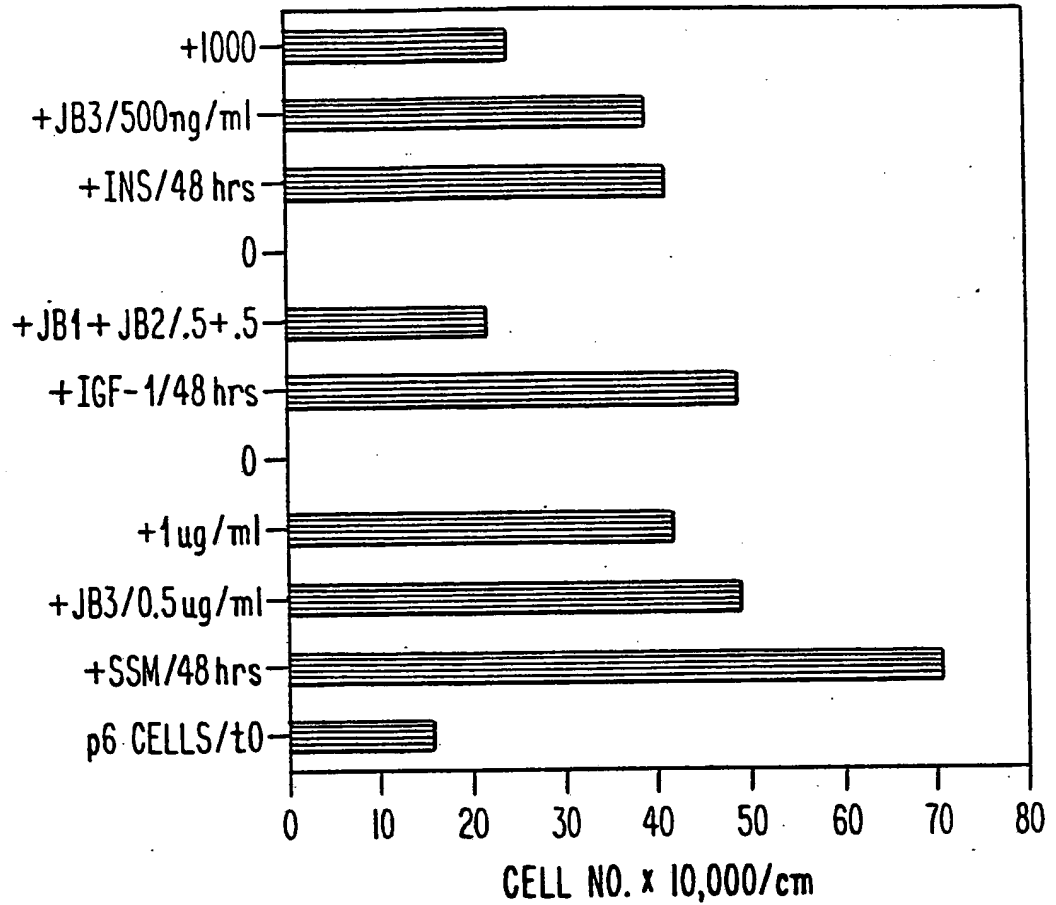
UNSTIMULATED  
INSULIN  
IGF-I  
JB-I  
500 ng/ml  
JB-I  
1000 ng/ml

FIG. 3

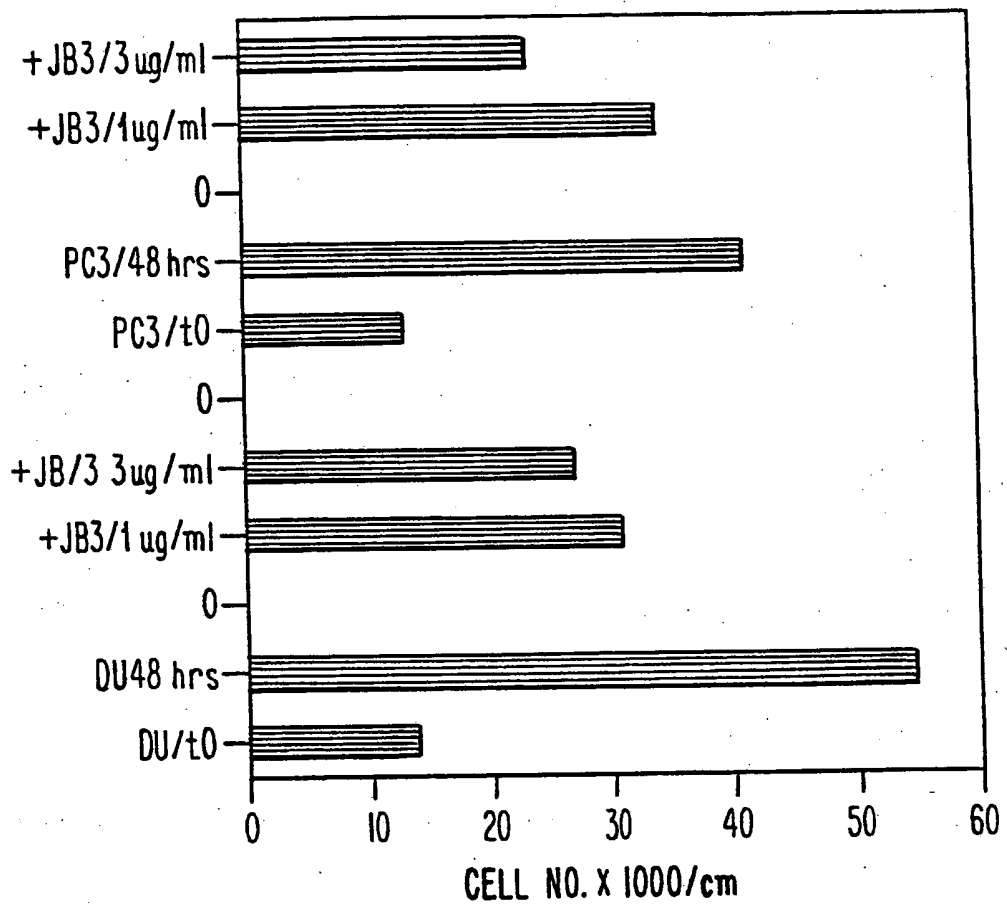
SUBSTITUTE SHEET



***Fig. 5***



***Fig. 7***



***Fig. 8***

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/04329

| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>IPC(5) :A61K 37/02; CO7K 5/10, 7/06, 7/08, 7/10<br>US CL :530/324, 327, 328 ; 514/12, 14, 15, 18<br>According to International Patent Classification (IPC) or to both national classification and IPC  |  |   |
|--|--|---|
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>U.S. : 530/324, 327, 328 ; 514/12, 14, 15, 18<br>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched<br>Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br>Cas, Aps, File Reg.  |  |   |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>  |  |   |
| Category*  | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No.   |
| X<br>Y   | US,A, 5,070,075 (Rotwein et al) 03 December 1991, see entire document.             | <u>1-7</u><br>8-22  |
| X<br>Y   | US, A, 5,077,276 (Ballard et al) 31 December 1991, see entire document.            | <u>1-7</u><br>8-22  |
| Y  | US,A 5,093, 317 (Lewis et al) 03 March 1992, see entire document.                  | 14-22   |
| X<br>Y   | WO, A, 89/05822 (Ballard et al) 29 June 1989, see entire document.                 | <u>1-7</u><br>8-22  |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.  |  |   |
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| Date of the actual completion of the international search<br>24 July 1993  |  | Date of mailing of the international search report<br>AUG 02 1993   |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. NOT APPLICABLE  |  | Authorized officer<br>S.G. MARSHALL<br>Telephone No. (703) 308-0196 |

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